

## **Microbiological Characterization of End-User Water at the MBL**

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Culture methods and molecular tools were used to characterize the microbial ecology of end-user water samples at the Marine Biological Laboratory in Woods Hole, Massachusetts. In this study, the relative abundance of targeted microbial groups was determined by CARD-FISH based on their presence on plate cultures or 16S rRNA sequence libraries. A recently reported quantitative-PCR method all-bacterial load was employed on parallel samples, and the results were compared to direct microscopic counts of DAPI-stained and EUB338 stained cells.

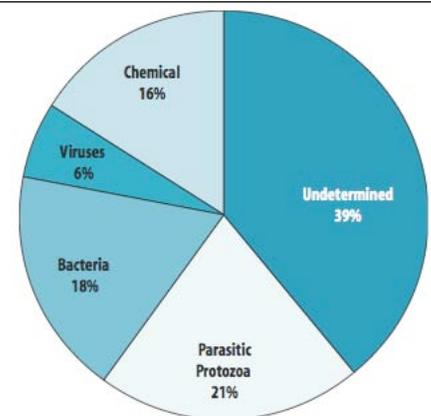
## Background and Significance

The United Nations stated in a 2003 report that clean water scarcity is the biggest challenge of the 21st century, and has placed protection of drinking water supplies at the forefront of the Millennium Development Goals (1). Furthermore, the World Health Organization has determined that current methods for water quality monitoring are inadequate for assessing the microbiological safety of drinking water (2). Microbial communities are known to exist throughout water distribution systems despite disinfection practices; however, current EPA-approved monitoring techniques provide no particular identification of the organisms present, and therefore the diverse consortium of microbes to which humans are exposed through drinking water is not well characterized.

Specific microbial pathogens have been shown to cause ~40% of investigated waterborne disease outbreaks, yet almost 40% of recent US waterborne disease outbreaks are of unknown origin (1) (Figure 1). The current standard in the US for assessing the microbiological safety of water is enumeration of heterotrophic thermotolerant organisms (termed fecal coliform bacteria) by classical microbiological plate-counting (2, 3). The rationale is that these indicator species can be used as a surrogate for the presence of enteric pathogens, and that microbial quality control is achieved by setting a maximum coliform count that water-

providing utilities must meet. However, such assays do not account for various microorganisms that may affect human health. In fact, it is now recognized by the World Health Organization and the Environmental Protection Agency that culture-based methods generally detect less than one percent of microbes present in a given sample (4, 5). Examples of undetected organisms include cyanobacteria that release neuro- and hepato-toxins into source water (6-8), as well as chlorine-resistant opportunistic pathogens of the mycobacterium group that may persist in water delivery systems (9-11). Other currently uncharacterized organisms may also have impacts on water users. This disparity and lack of understanding is the basis for the study described herein.

**Figure 1 - Causes of Waterborne Disease Outbreaks in the USA, 1991-2000**



## Community Analysis Methods

1. Isolation of plate-cultured heterotrophs. Water samples for plating were collected from 2 drinking fountains in LOEB Hall. 1 mL, 500 ul, 250 ul, and 100ul were added directly to R2A agar media plates, spread with an ethanol flamed glass rod, and incubated at 30 C for 7 days. Colonies were periodically isolated as they appeared by restreaking, and each isolate was PCR-amplified for the 16S rRNA gene using primers 8F and 1492 R.

2. Total DNA extraction, PCR Amplification, and Sequencing of 16S rRNA genes. Microbial community composition of water samples from 3 end-user sites at the MBL was surveyed. Water samples were collected from a drinking fountain (DFW2), a tap source (EBW), and a biofilm enrichment experiment (BF). 500 ml of each water sample was concentrated on 0.2-micron filters through sterile filtration devices. Cells were scraped from biofilm experiment slides and suspended in 200ul of biofilm vessel water. Total DNA was extracted from filters by bead-beating lysis with subsequent DNA purification by the MoBio soil DNA extraction kit. PCR with universal primers (515F and 1391R), cloning with the TOPO TA kit, and sequencing of transformant plasmid DNA was carried out for each sample. Sequence libraries generated were aligned by greengenes NAST aligner and phylogenetically analyzed using the ARB software package.

3. Abundance assays: QPCR bacterial load assay and DAPI/CARDFISH direct microscopic counts.

- A. Total microbial load was determined by QPCR assays that target a broad spectrum of bacterial groups by using a primer set directed at a highly conserved 331 F & 797R 16S rRNA region (12). Total DNA was extracted (described above) from 500, 250, and 100 ml volumes. Full strength and 1:10 dilutions of DNA were used in PCR reactions (10ul Sybergreen Supermix, 1ul 331F, 1ul 797R, 7 ul H<sub>2</sub>O, and 1 ul template).
- B. Total cell number was determined by direct microscopic counts of DAPI-stained cells. Water samples were taken in 50 ml volumes, fixed in 1% formaldehyde, and concentrated onto Millipore 0.2 um polycarbonate filters. Filters were sections, stained with 30 ul 1ug/ul DAPI solution for 5 minutes, washed in autoclaved milliQ water 1 minute, washed in ethanol 1 minute and mounted on microscope slides in a 4:1 citiflour: vectashield solution. Fields were counted under 400X and 1000X magnification.
- C. Relative abundance of target microbial groups was determined by Catalyzed Reporter Deposition Fluorescence In-Situ Hybridization (CARDFISH). Sequence library information and culture isolates provided insight into microbial community composition, and these organisms were targeted by group-specific probes. Probes used included Eub338, Non338, Gam42, and Bet42 (see laboratory manual for protocol).

## Results

1. Isolation of plate-cultured heterotrophs. 9 sequences from isolated colonies were successfully obtained. The sequences were aligned by the NAST aligner and inserted into the greengenes ARB database. A maximum likelihood phylogenetic tree was constructed (Figure 2). The following taxonomy was obtained from greengenes by uploading the fasta file and classifying the batch:

bigwhite	Betaproteobacteria	Burkholderiales	Acidovorax
bigwhite2	Betaproteobacteria	Burkholderiales	Acidovorax
lightorange	Alphaproteobacteria	Sphingomonadales	Novosphingobium
pink	Alphaproteobacteria	Rhizobiales	Methylobacterium
smoothorange	Alphaproteobacteria	Sphingomonadales	Porphyrobacter
yellow	Actinobacteridae	Actinomycetales	Citricoccus
yellow2	Actinobacteridae	Actinomycetales	Rathayibacter
yellow4	Actinobacteridae	Actinomycetales	Rathayibacter
yellow5	Actinobacteridae	Actinomycetales	Rathayibacter

2. Total DNA extraction, PCR Amplification, and Sequencing of 16S rRNA genes. Three different water samples were used in this study to generate 16S rRNA sequence libraries. The first sample was obtained from a drinking water fountain (DFW2), the second from a bathroom tap in a residence hall (EBW), and the third from a biofilm (BF) enrichment experiment from the DF2 water fountain. Total DNA was extracted (Figure 3) and libraries of 96, 48, and 48 clones (respectively) were generated. The sequences as analyzed by the ARB phylogenetic software package fell mostly within the Proteobacteria supergroup, along with some representatives from the Actinobacteria (Figure 4).

3. Abundance assays: QPCR bacterial load assay and DAPI/CARDFISH direct microscopic counts.

Quantitative PCR was used to assay total 16S rDNA copy number with primers 331F and 797R. The measured cycle threshold was compared to a standard curve of E.coli genomic DNA that corresponded to cell number using the conversion fraction  $2.38 \text{ ng DNA} = 4.8 \times 10^5 \text{ cells}$  (Figure 5). Thereby total bacterial cell load was back-calculated based on 16S rRNA gene amplification. (Figure 6).

Four water sources were assayed for total cell counts by direct microscopic visualization of DAPI stained cells. CARDFISH was used to assess the relative distribution of two Proteobacterial groups. Total direct microscopic counts are summarized as DAPI counts in cells/ml and calculating

Eubacteria, Gammaproteobacteria, and Betaproteobacteria as percentages of total DAPI counts (Figure 7).

## **Conclusions**

The goal of this project was to determine the community composition of selected end-user water samples from the MBL, and to investigate relative abundance of target groups. Community composition, based on a limited sequence library size, was found to include members of the Proteobacteria supergroup, as well as representatives from the Actinobacteria. However, it was also possible to detect cells by staining with CARDFISH probes that target the Cytophaga and Planctomycetes groups (data not shown). These cells were very near the detection limit of CARDFISH. Nevertheless, they may constitute up to 1% of the water community and therefore the generation of additional clone libraries to obtain greater sequence coverage is warranted.

Direct microscopic counts of fluorescently stained cells revealed differences in total bacterial cell load among water samples. The cell load ranged from  $1 \times 10^5$  in bottled water to  $3 \times 10^6$  in residence hall tap water. A QPCR assay was employed to attempt detection of this observation by an alternate method. On average the QPCR assay determined cell counts to be at least a factor of ten lower than direct counts. This is most likely attributable with the loss of some DNA during the extraction process. This assay needs to be further optimized to provide quantitative analyses of total bacterial cell load. At this time, it is useful for quantifying relative amounts of DNA from samples extracted by the same process.

Further experiments, had there been time, would include additional screening of clones for more adequate coverage of sequence diversity in water samples. Also, clone libraries could be generated for the additional water sources that were investigated by the CARDFISH assay (data not shown). The QPCR assay could also be tested with additional standard curves to optimize the concentration gradient that would give comparable results to direct count data.

## References Cited

1. Chlorine Chemistry Council (CCC), in *Drinking water chlorination: A review of disinfection practices and issues* (2003).
2. D. E. Burmaster, K. E. von Stackelberg, *Qual Assur* **1**, 192-206 (1992).
3. W. S. Cooter, *Environ Sci Technol* **38**, 5265-73 (2004).
4. R. I. Amann, W. Ludwig, K. H. Schleifer, *Microbiol Rev* **59**, 143-69 (1995).
5. *Assessing microbial safety of drinking water: improving approaches and methods* (OECD, Paris, 2003).
6. W. Schmidt, H. Willmitzer, K. Bornmann, J. Pietsch, *Environ Toxicol* **17**, 375-85 (2002).
7. J. Rapala, K. Lahti, L. A. Räsänen, A. L. Esala, *et al.*, *Water Res* **36**, 2627-35 (2002).
8. J. M. Dos S Vieira, M. T. de P Azevedo, S. M. de Oliveira Azevedo, R. Y. Honda, B. Corrêa, *Toxicol* **45**, 901-9 (2005).
9. S. Goslee, E. Wolinsky, *Am Rev Respir Dis* **113**, 287-92 (1976).
10. E. D. Hilborn, T. C. Covert, M. A. Yakrus, S. I. Harris, *et al.*, *Appl Environ Microbiol* **72**, 5864-9 (2006).
11. M. J. Vaerewijck, G. Huys, J. C. Palomino, J. Swings, F. Portaels, *FEMS Microbiol Rev* **29**, 911-34 (2005).
12. M. A. Nadkarni, F. E. Martin, N. A. Jacques, N. Hunter, *Microbiology* **148**, 257-66 (2002).

Figure 2. Maximum Likelihood tree of colony sequences.

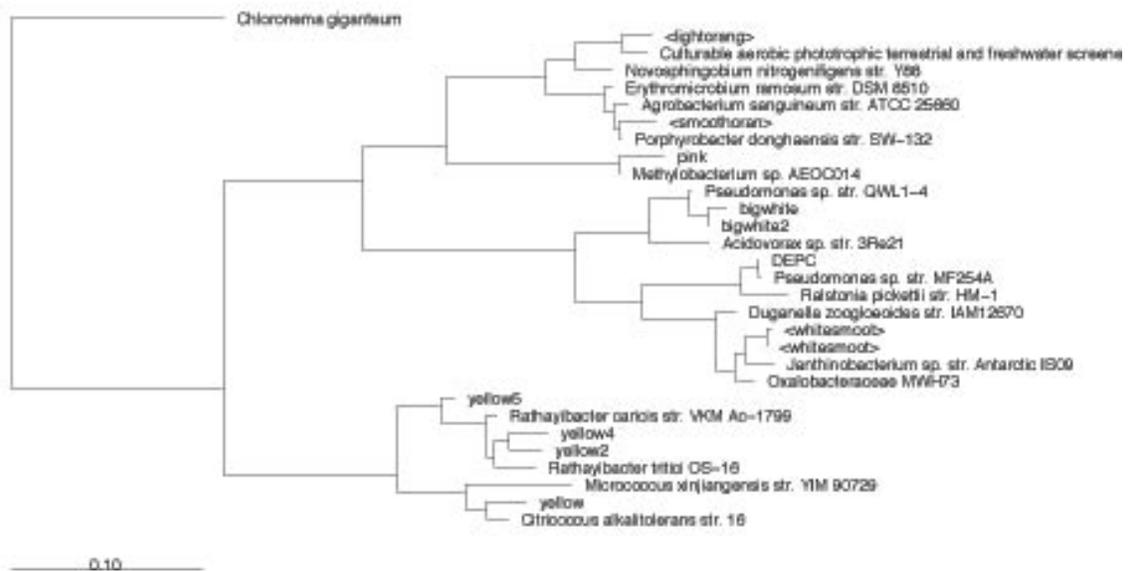
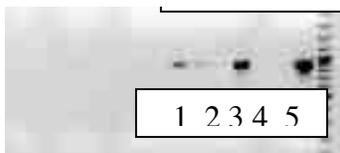


Figure 3. Extracted DFW2 DNA on 1% agarose gel. (EBW and BF DNA not shown)



1. DFW2 DNA 250ml
2. DFW2 DNA 250 ml 1:10 dilution
3. Poison control (2 ul DFW2 DNA + 1 ul *E.coli* genomic)
4. Negative NF H2O
5. Positive *E.coli*

Figure 4. Maximum Likelihood tree of selected sequences from BF, EBW, and DFW2 clone libraries.

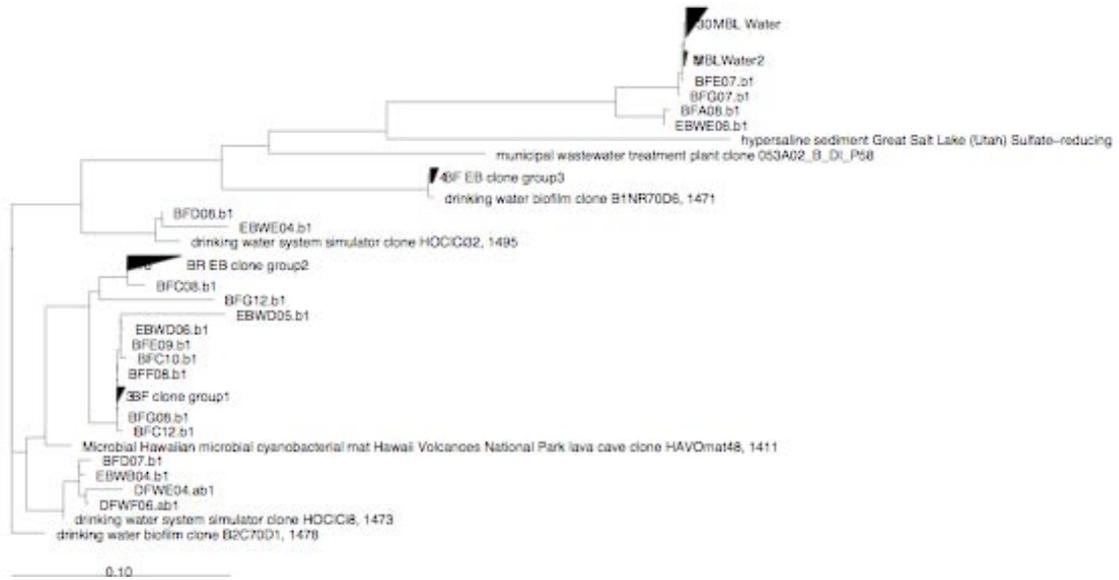


Figure 5. Standard QPCR curve using *E. coli* genomic DNA.

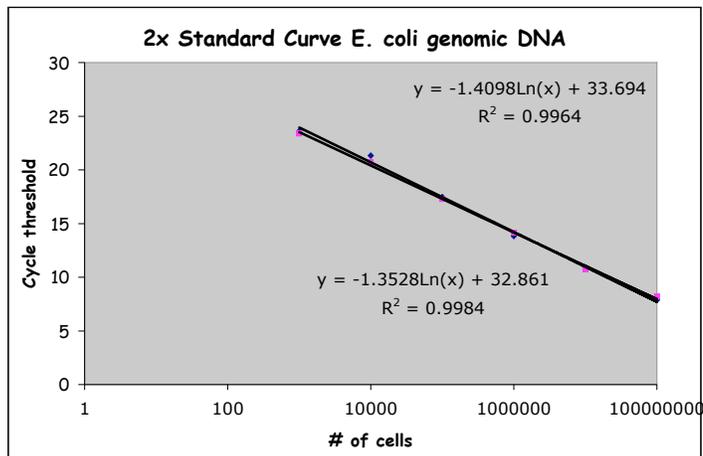


Figure 6. QPCR assay results corrected for volume of water in each sample.

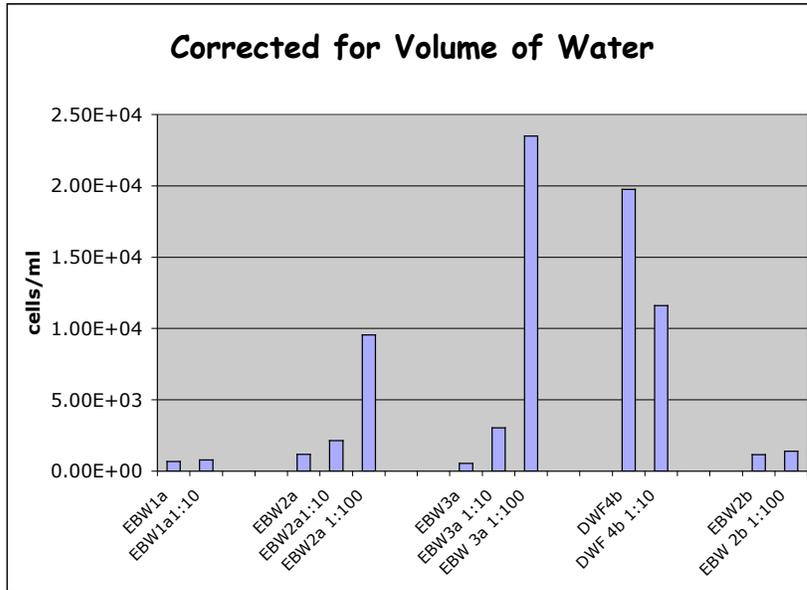


Figure 7. Summary of direct counts by fluorescence microscopy.

<b>DFW2</b>	
<b>DAPI</b>	<b>2.4 * 10<sup>5</sup></b>
<b>EUB</b>	<b>78%</b>
<b>Gamma</b>	<b>1.89%</b>
<b>Beta</b>	<b>9.02%</b>
<b>DFW1</b>	
<b>DAPI</b>	<b>1 * 10<sup>5</sup></b>
<b>Eub</b>	<b>72%</b>
<b>Gamma</b>	<b>24.55%</b>
<b>Beta</b>	<b>13.50%</b>
<b>WCW</b>	
<b>DAPI</b>	<b>3 * 10<sup>5</sup></b>
<b>Eub</b>	<b>86%</b>
<b>Gamma</b>	<b>1.38%</b>
<b>Beta</b>	<b>56.65%</b>
<b>EBW</b>	
<b>DAPI</b>	<b>3 * 10<sup>6</sup></b>
<b>Eub</b>	<b>76%</b>
<b>Gamma</b>	<b>2.43%</b>
<b>Beta</b>	<b>15.39%</b>